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Percent Charging of Transfer Ribonucleic Acid and Levels of ppGpp and pppGpp in Dormant and Germinated Spores of *Bacillus megaterium*

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The levels of transfer ribonucleic acids (tRNAs) specific for 14 amino acids were almost identical in dormant spores and in spores germinated from 6 to 75 min. Germinated spore tRNAs specific for all amino acids tested were between 63 and 93% charged, and there was no significant change in this value from 6 to 75 min of germination. In contrast, tRNAs isolated from dormant spores specific for nine different amino acids were almost completely($>93\%$) uncharged. However, some dormant spore tRNAs, i.e., those for arginine, histidine, isoleucine, and valine, showed significant (21 to 72%) levels of aminoacylation. Dormant spores contained no detectable guanosine penta- (pppGpp), tetra- (ppGpp), or triphosphate (GTP). However, these nucleotides appeared in the first minutes of germination, and thereafter all increased in parallel with a ratio of pppGpp plus ppGpp to GTP of 0.07 to 0.11, which is characteristic of unstarved vegetative cells.

Aminoacyl-transfer ribonucleic acid (tRNA) is a required substrate for the process of protein synthesis. The percentage of bacterial tRNA charged under normal conditions in vivo ranges from 60 to 90%, although some charged tRNA is not aminoacylated (6, 12, 27). The level of aminoacyl-tRNA in bacteria has been implicated in control of amino acid biosynthetic operons (12) and regulation of intracellular proteolysis (8). A low level of charging of one or more tRNAs is also thought to initiate the stringent response by increasing the level of the unusual guanosine nucleotides—guanosine pentaphosphate (pppGpp) and guanosine tetraphosphate (ppGpp) (3, 7, 9, 15).

Dormant spores of *Bacillus* species have normal levels of tRNA (26) but carry out no detectable protein synthesis (11). In addition, spores have extremely low levels of adenosine triphosphate (ATP) and most amino acids (16, 21). These observations, together with the absence of the 3'-terminal adenosine monophosphate (AMP) residue from one-third of the tRNA in *B. megaterium* spores (23), suggested that much dormant spore tRNA might be uncharged despite the fact that aminoacyl-tRNA synthetases are present (25). On the other hand, aminoacyl-tRNA must be present in germinated spores, since protein synthesis begins during the first minutes of germination (11). Nevertheless, a number of events occurring in these first minutes of germination suggest that at this time the percentage of charging of some tRNAs might be lower than during normal vegetative growth. These properties include the rapid proteolysis which occurs (16, 17) and the low energy charge of the adenine nucleotide pool (1, 21). These observations prompted me to examine the levels of charged and uncharged tRNAs in dormant and germinated spores of *B. megaterium*. Levels of pppGpp and ppGpp were also determined, since they are thought to be dependent on the level of uncharged tRNA.

MATERIALS AND METHODS

Substrates. Tritium-labeled amino acids were obtained from the New England Nuclear Corp. and were stored at 4 C. All amino acids were used within 40 days of purchase, and all but one were $>92\%$ pure as determined by paper chromatography in butanol-acetic acid-water (10:2:5). Proline was only 86% pure. Sources of ATP, α-[^{32}P]ATP, AMP, guanosine triphosphate (GTP), guanosine monophosphate (GMP), [^{32}P]orthophosphate, polyethylenimine-cellulose chromatography sheets, and yeast tRNA-C-C were described previously (21, 23).

Production and germination of spores. Dormant and ^{32}P-labeled spores of *Bacillus megaterium* QM B1551 were grown in supplemented nutrient broth, harvested, cleaned, lyophilized, and stored as de-

scribed previously (20, 21). The specific radioactivity of ^{32}P-labeled spores was also determined as described previously (21). All spore preparations were free from cells and cell debris, and >98% of the spores were refractile as judged in the phase-contrast microscope. For studies on tRNA, spores were germinated after heat treatment (10 min, 60 C) of spores (25 mg [dry weight/ml]) in water and subsequent cooling. Germination was at 30 C with a spore concentration of 0.5 mg/ml in Spizizen medium without added Casamino Acids (24). For studies of nucleotide levels, unlabeled dormant spores were germinated at 2.5 mg/ml under identical conditions but with the medium modified to contain 1 mM [^{32}P]phosphate (100 μCi/μmol) and 10 mM tris(hydroxymethyl)aminomethane (Tris)-hydrochloride (pH 7.4). Initiation of germination in both media was >95% complete by 15 min as determined in the phase-contrast microscope, and the low amount of phosphate in the modified medium gave no significant reduction in rates of protein and RNA synthesis until 100 min of germination.

Extraction of nucleotides. At various times samples (100 μliters) from germinating cultures were diluted with an equal volume of cold 2 M formic acid (3). After 45 min at 4 C the mix was centrifuged and the supernatant fraction was used for determination of nucleotide content. ^{32}P-labeled dormant spores (30 mg) were also extracted with 0.5 ml of 1 M formic acid, as described above, after dry rupture of the spores in a dental amalgamator (Wig-L-Bug) with glass beads as described previously (18).

Separation and quantitation of nucleotides. Unlabeled ATP and GTP were added as markers to small volumes of formic acid extracts, and ATP, pppGpp, ppGpp, and GTP were separated by thin-layer chromatography on polyethylenimine-cellulose. After application of samples, the chromatography sheets were pre-run in methanol-water (2:1) in the direction perpendicular to the direction of chromatography. The chromatogram was then developed with 1.5 M potassium phosphate (pH 3.4) (3). Nucleotides were located both by their absorption under ultraviolet light and by autoradiography, and radioactive spots were cut out and counted in a gas flow counter as described previously (21). ATP and GTP were identified by using the unlabeled markers, whereas ppGpp and pppGpp were identified by using their published R_f values (3). Only two spots were found in the region of the chromatogram where ppGpp and pppGpp were expected. Furthermore, isolation, purification, and hydrolysis (1 N HCl, 100 C, 10 min) of a large amount of putative [^{32}P]ppGpp (3) released 50% of the label as phosphate as would be expected from hydrolysis of the two pyrophosphate linkages. AMP and GMP were also separated by thin-layer chromatography as described previously (21) and quantitated as described above. Calculations of nucleotide levels took into account the number of phosphates in the various compounds. As previously described (22), values were also corrected for the absence of nucleotide biosynthesis early in germination, since there is probably little incorporation of label into the 5′-α position of nucleotides during this time.

Enzyme preparation and assay. Aminoacyl-tRNA synthetases from *B. megaterium* QM B1551 vegetative cells were extracted, purified, stored, and assayed as described by Vold and her co-workers (25, 26). The purification procedure does not separate individual synthetases, but does remove amino acids, nucleases, and tRNAs. tRNA nucleotidyltransferase was assayed as described previously (23), and protein was determined by the Lowry procedure (14).

Extraction and periodate oxidation of nucleic acid. Charged tRNA was determined as the percentage of tRNA acceptor activity resistant to periodate oxidation at 37 C. This procedure, as well as the extraction and periodate oxidation of tRNA, was adapted from that of Folk and Berg (6) as modified by Lewis and Ames (12), and is outlined in Scheme 1. Prewarmed (30 C) 50% trichloroacetic acid (33 ml) was added to germinating spores (300 ml) as described (12). The culture was then shaken, treated with sodium dodecyl sulfate, chilled, and centrifuged (6). The pellet was immediately frozen in dry ice-ethanol and freeze-dried, and the dried spores then broken in a dental amalgamator (Wig-L-Bug) (18). The resulting powder was suspended in 3.5 ml of 0.25 M sodium acetate (pH 7.0)-0.05% SDS-1 mM ethylenediaminetetraacetate (final pH ~5.0) (12). Subsequent phenol extraction, precipitation, periodate and mock periodate treatment (each on one-half of every extract), deacylation, and storage of nucleic acids were carried out as described by Lewis and Ames (Scheme 1) (12). Dry, dormant spores were also ruptured in a dental amalgamator, and the resulting powder was suspended in 25 ml of cold 5% trichloroacetic acid. After 20 min at 4 C, the mixture was centrifuged and the pellet was treated as described above (steps 3 through 10, Scheme 1).

Deacylated tRNA was prepared from nucleic acid extracted from spores germinated for 20 min and purified through the first ethanol precipitation as described above (steps 1 through 5, Scheme 1). The tRNA was then deacylated (step 9, Scheme 1), and subsequently samples were subjected to periodate or mock periodate oxidation (steps 6 through 8 and 10, Scheme 1) (12).

Assay of tRNA content in extracted and treated nucleic acids. The content of individual amino acid-accepting tRNAs was assayed in 100-μliter incubation mixtures using the concentrations of ATP, $MgCl_2$ or Mg acetate, buffer, 2-mercaptoethanol, and salt established by Vold and Minatogawa as optimal for different aminoacyl-tRNA synthetases from *B. subtilis* (26). Labeled amino acid was present at 10 to 12.5 μM (about 1,000 counts per min per pmol), and 1 to 2 absorbancy units (at 260 nm) (A_{260}) of nucleic acid and 5 to 20 μliters of aminoacyl-tRNA synthetase were added. After incubation at 37 C for 10 to 20 min, aminoacyl-tRNA was collected and quantitated as previously described (23). For each amino acid tested, assays were performed with two different incubation times and with different amounts of aminoacyl-tRNA synthetase to ensure that maximal aminoacylation was reached and maintained. Most samples were also tested at several concentrations of nucleic acid. All

SCHEME 1. *Procedure for extraction and treatment of nucleic acid from B. megaterium spores*[a]

Step No.	Treatment
1	Trichloroacetic acid was added to culture of spores, SDS[b] was added, and the culture was chilled and centrifuged.
2	Pellet from step 1 was lyophilized, and dry spores were ruptured in dental amalgamator.
3	Residue from step 2 was suspended in sodium acetate (pH 7.0)-SDS-EDTA.[c] Final pH of this suspension was ~5.0.
4	Suspension from step 3 was extracted with phenol, layers were separated by centrifugation, and the phenol layer was reextracted with 0.25 M sodium acetate (pH 5.0).
5	Aqueous layers from step 4 were pooled and precipitated with 2 volumes of ethanol (−20 C).
6	Precipitate from step 5 was washed two times with ethanol and dissolved in 0.1 M sodium acetate (pH 4.6).
7a	Sodium periodate was added to one-half of the solution from step 6, and the resulting solution was incubated for 30 min at 20 C in the dark (periodate treatment).
7b	One-half of the solution from step 6 was incubated for 30 min at 20 C in the dark (mock periodate treatment).
8	Solution from step 7 was made 0.5 M in NaCl, and nucleic acids were precipitated with 2 volumes of ethanol. The precipitate was washed two times with ethanol and dissolved in 0.1 M sodium acetate (pH 4.6) containing 0.1 M ethylene glycol. After 10 min at 20 C in the dark, the solution was made 0.5 M in NaCl and nucleic acids were precipitated with 2 volumes of ethanol (−20 C).
9	Pellet from step 8 was washed two times with ethanol and then dissolved in 1.8 M Tris-hydrochloride (pH 8.2) (deacylation step). After 2 h at 37 C, nucleic acids were precipitated with 2 volumes of ethanol (−20 C).
10	Pellet from step 9 was washed two times with ethanol, dried under a stream of air, dissolved in water, and frozen.

[a] Further details of individual steps are given in Materials and Methods.
[b] Sodium dodecyl sulfate.
[c] Ethylenediaminetetraacetic acid.

nucleic acid samples were assayed in triplicate, and values were corrected for blanks obtained in incubations minus nucleic acid, minus aminoacyl-tRNA synthetase, or minus both. Generally blank values were 50 to 100 counts/min, whereas values in the complete system were 700 to 3,000 counts/min. Values for the percentage of charged tRNA in each extract were calculated from the ratio of acceptor activity (picomole/A_{260}) in the periodate-treated sample to that in the mock periodate-treated sample.

Larger amounts of aminoacyl-tRNA were synthesized in reaction mixtures identical to those described above, but with 10 to 20 times the volume. Nucleic acids were precipitated with trichloroacetic acid (final concentration 5%) and centrifuged, and the pellet was washed five times with ethanol-water (2:1) and dissolved in 0.1 M sodium acetate (pH 4.6) just prior to use.

Determinations of amino acids bound to dormant spore tRNA. Nucleic acids were extracted from dry dormant spores (7 g) and purified through the first ethanol precipitation (steps 1 through 5, Scheme 1). The pellet was dissolved in 40 ml of 0.1 M sodium acetate (pH 4.6) and precipitated with ethanol, and the pellet was dried under a stream of air. Approximately equal amounts of this pellet were dissolved in 10 ml of either 0.1 M sodium acetate (pH 4.6) (mock-deacylated sample) or 1.8 M Tris-hydrochloride (pH 8.2) (deacylated sample), and two small volumes of the mock deacylated sample were taken through the periodate and mock periodate treatment (steps 6 through 10, Scheme 1). The rest of the samples were incubated at either 4 C (mock-deacylated sample) or 37 C (deacylated sample) for 1.5 h. The nucleic acid was then precipitated with ethanol, and the supernatant fraction was flash-evaporated. The residue from each sample was dissolved in 50 ml of water, 10 ml of 1.5 M Tris-hydrochloride (pH 8.2) was added to the mock-deacylated sample, and 10 ml of 0.1 M sodium acetate (pH 4.6) was added to the deacylated sample. After the pH was adjusted to 1.7 with HCl, these solutions were loaded onto Dowex 50 columns (hydrogen form; 1.2 by 20 cm), and the columns were washed with 20 ml of water. Amino acids were eluted with 100 ml of 3 M NH_4OH, and the eluates were flash-evaporated. Samples of the dissolved residue were analyzed on an amino acid analyzer. The Dowex chromatography procedure gave recoveries of >90% for alanine, arginine, glutamate, leucine, and valine.

RESULTS

Levels of specific tRNAs in dormant and germinated spores. Several workers have shown that there is little change in the relative amounts of different amino acid-accepting tRNAs during growth and sporulation in *Bacillus* species, although significant differences in a few species have been noted (10, 26).

Likewise, I also observed little difference (<15%) in the absolute levels of 14 amino acid-accepting tRNAs from either dormant spores or spores germinated from 6 to 75 min (Table 1). Assuming that 14% of the nucleic acid extracted from dormant spores is tRNA (4), it can be calculated that the values obtained for dormant spore tRNA are similar to those reported previously in both this organism (5, 23) and *B. megaterium* KM (26). Since the latter studies utilized purified tRNA and in one case purified aminoacyl-tRNA synthetases, it appears that the use of unfractionated nucleic acid and crude synthetases gave accurate values for the amino acid-acceptor activity of spore tRNA.

It was at first surprising that dormant spore tRNA did not show significantly lower levels of a number of amino acid-accepting species, since 35% of dormant spore tRNA contains defective 3'-termini and this value is as high as 90% for some species (23). However, all aminoacyl-tRNA synthetase preparations used in this study were found to contain significant amounts of tRNA nucleotidyltransferase relative to amounts of aminoacyl-tRNA synthetase (Table 2). Indeed, the level of tRNA nucleotidyltransferase present in the aminoacylation reactions was sufficient to repair all defective 3'-termini at least 10 times over. This value was calculated using the data in Table 2, the known conditions of the aminoacylation reaction, and a value of 35% for the percentage of defective 3'-termini (23). Any termini not repaired due to absence of a cytidine monophosphate residue in addition to the 3'-terminal AMP would amount to only 4 to 8% of the total tRNA (23), a value probably below the accuracy of the data.

TABLE 1. *Amino acid-acceptor activities of tRNA species from dormant and germinated spores of B. megaterium*[a]

Amino acid	Amount of amino acid accepted (pmol/A_{260})			
	Dormant spores[b]	Germinated spores[b]		
		6 min[c]	20 min[c]	75 min[c]
Alanine	4.1	4.4	4.1	4.5
Arginine	5.2	5.1	5.0	5.2
Glutamic acid	8.8	8.2	8.6	9.2
Glycine	5.5	5.5	5.4	5.5
Histidine	2.6	2.6	2.5	2.5
Isoleucine	4.9	5.2	5.3	4.9
Leucine	7.2	7.3	7.8	7.9
Lysine	4.3	4.7	4.2	4.7
Methionine	7.4	7.1	6.4	6.4
Phenylalanine	4.4	4.1	4.1	4.2
Proline	3.3	3.5	3.2	3.3
Serine	5.4	6.0	5.8	5.9
Tyrosine	3.9	3.6	4.0	3.9
Valine	6.3	6.0	6.5	6.5

[a] Nucleic acids were extracted, purified, subjected to the mock periodate treatment, deacylated, and stored as described in Materials and Methods and Scheme 1 (steps 1 through 6, 7b through 10). Amino acid acceptor activities are averages of two separate determinations and values were ± 9%.

[b] Source of nucleic acid.

[c] Minutes of germination.

Percentage of tRNA charged in dormant and germinated spores. Although the relative acceptor activities for different amino acids were very similar in tRNA from dormant and germinated spores, the percentage of the tRNA charged in these two stages was extremely different. Germinated spore tRNAs for all amino acids tested were between 63 and 93% charged (Table 3), as observed in *Salmonella typhimurium* and *Escherichia coli* (6, 12, 27). For no amino acid tested did the percentage of charged tRNA increase significantly (more than 8%) from 6 to 75 min of germination, and values at the different times varied at most by 12%, which is approximately the experimental error. Dormant spores, on the other hand, contained extremely low levels (<8%) of charged tRNA for at least nine amino acids (Table 3). However, four tRNAs from dormant spores were charged to significant levels, and $tRNA^{val}$ was charged to a similar extent in both dormant and germinated spores.

The recovery of labeled aminoacyl-tRNAs added to spore extracts prior to phenol extraction (at step 3, Scheme 1) was between 90 and 95% for the four aminoacyl-tRNAs tested (Table 4). Levels of charging have not been corrected for this small amount of loss. Similarly, the deacylation procedure (step 9, Scheme 1) released >96% of these four amino acids from tRNA (Table 4), and rendered tRNAs for all amino acids tested >92% sensitive to oxidation by periodate (column 4, Table 5).

Aminoacyl-tRNAs in dormant spores. Although the low level of charged tRNA in dormant spores was not surprising in view of their lack of ATP (21), it was surprising that several tRNA species showed high levels of charging. Therefore, this finding was examined in more detail. The high levels of charging were not due to resistance of individual tRNA species to the periodate treatment, since the five tRNA species in question (along with all others tested) were all destroyed (>92%) by periodate treatment following deacylation (column 4, Table 5). Similarly, it is extremely unlikely that acylation could have occurred during breakage of

TABLE 2. *Specific activities of enzymes in aminoacyl-tRNA synthetase preparations*[a]

Preparation of aminoacyl-tRNA synthetase	Protein mg/ml	Sp act (pmol/10 min/20 μliters)	
		Leucyl-tRNA synthetase	tRNA nucleotidyltransferase
I	4.3	37	210 (140)
II	3.3	29	130 (86)

[a] Aminoacyl-tRNA synthetase preparations were isolated and enzymes were assayed as described in Materials and Methods. Values in parentheses were obtained by assay with Tris-hydrochloride (pH 7.4).

TABLE 3. *Percent charging of different amino acid-accepting tRNAs from dormant and germinated spores of B. megaterium*[a]

Amino acid	tRNA charged (%)			
	Dormant spore[b]	Germinated spore[b]		
		6 min[c]	20 min[c]	75 min[c]
Alanine	<5	81	83	76
Arginine	21	85	87	84
Glutamic acid	7	64	72	63
Glycine	<5	84		82
Histidine	34	77	83	79
Isoleucine	33	93	78	81
Leucine	<5	90	85	86
Lysine	6	84	84	78
Methionine	<5	91	86	80
Phenylalanine	12	83		82
Proline	<5	93	93	83
Serine	<5	76		77
Tyrosine	<5	88	88	85
Valine	72	88	84	81
Avg	~15	84.0	83.9	79.8

[a] Nucleic acids were extracted and treated, and the level of charging was determined as described. Values were ± 6%.
[b] Source of nucleic acid.
[c] Minutes of germination.

spores and extraction of the tRNA, since this process has previously been shown to allow no ATP production (23).

However, it was possible that the charged tRNA in dormant spores might not be aminoacyl-tRNA, but rather some labile moiety other than an amino acid protecting the 3'-terminus of tRNA against periodate oxidation, as has been found in *E. coli* (27). Therefore, I analyzed the amino acids bound to dormant spore tRNA (column 3, Table 5). The amounts of arginine, isoleucine, and valine released from spore tRNA under deacylating conditions were in excellent agreement with the amount of aminoacyl-tRNA in spores calculated from the levels of charging (columns 2 and 3, Table 5). Therefore, these three aminoacyl-tRNAs are almost certainly present in dormant spores. Unfortunately, the amounts of histidine and phenylalanine expected were too low to enable any conclusions about the presence of these aminoacyl-tRNAs in dormant spores.

Levels of pppGpp, ppGpp, and GTP in dormant and germinating spores. Despite the presence of high levels of uncharged tRNA, dormant spores contained no detectable pppGpp and ppGpp (Table 6). In agreement with previous work, ATP and GTP levels were also extremely low in dormant spores, and significant amounts of AMP and GMP were present (21) (Table 6). Since GTP and ATP were absent from dormant spores, it was not completely surprising that ppGpp and pppGpp were also absent. However, these data suggest that during sporulation the shutoff in protein synthesis and the increase in levels of uncharged tRNA in the developing spore must occur in such a fashion as to prevent the corresponding accumulation of ppGpp and pppGpp (9, 15). A possible mechanism for regulating all three of these processes may be the dramatic decrease in the ATP pool which must occur in the developing spore during sporulation (21).

pppGpp and ppGpp were detectable by the

TABLE 4. *Recovery and subsequent deacylation of aminoacyl-tRNA added to spore preparations*[a]

Aminoacyl-tRNA added	Recovered (%)	Resistant to deacylation (%)
Alanine[b]	92	<1
Glycine[c]	90	<1
Methionine[b]	95	2
Valine[c]	91	3

[a] Labeled aminoacyl-tRNA (~20 pmol) was added to disrupted dormant spores just after suspension of the dry powder in trichloroacetic acid; addition to preparations from germinated spores was also to the suspension of disrupted spores (step 3, Scheme 1). Samples were treated further through the mock periodate or periodate steps (steps 4 through 8, Scheme 1) and subsequently deacylated at pH 8.2 (steps 9 through 10, Scheme 1) (also see Materials and Methods). Recoveries were calculated by comparing the total acid-precipitable radioactivity of the initial solution and the solution in step 6 (Scheme 1), and the specific radioactivities (acid precipitable counts per minute per A_{260}) of the latter solution and the average of those at the end of step 8 (Scheme 1). The amount of aminoacyl-tRNA resistant to deacylation was determined similarly.
[b] Aminoacyl-tRNA added to germinated spore preparations.
[c] Aminoacyl-tRNA added to dormant spore preparations.

TABLE 5. *Characterization of charged tRNAs of dormant spores of B. megaterium*[a]

Amino acid	Total acceptor activity (pmol/A_{260})	Charged[b] tRNA (pmol/A_{260})	Amino acid bound to tRNA (pmol/A_{260})	Acceptor activity resistant to periodate (%)
Arginine	5.0	1.0	0.8 (0.3)	1
Isoleucine	5.1	1.3	1.2 (0.4)	8
Valine	6.5	4.9	5.3 (0.6)	2
All other amino acids tested			Each <0.4 (less than 1.0 except serine which was 2.9)	<4

[a] Nucleic acids were extracted, purified, and treated and amino acids were purified as described in Materials and Methods. Total acceptor activity and charged tRNA were determined as described, as were the amino acids bound to tRNA. Amino acids bound to tRNA were calculated as the difference in amino acid levels between the deacylated and mock-deacylated samples. Values in parentheses are amino acid levels in the mock-deacylated sample. Acceptor activity resistant to periodate oxidation was calculated as the ratio of the acceptor activity of deacylated tRNA subjected to periodate oxidation over the acceptor activity of the same tRNA given the mock periodate treatment.

[b] Corrected for tRNA resistant to periodate.

TABLE 6. *Levels of guanine and adenine nucleotides in dormant spores of B. megaterium*

Nucleotide	Level (nmol/g of dry spores)
pppGpp	<0.6
ppGpp	<0.6
GTP	<1
GMP	123
ATP	6
AMP	1,120

[a] ^{32}P-labeled dormant spores were broken and extracted, and nucleotides were determined as described in Material and Methods.

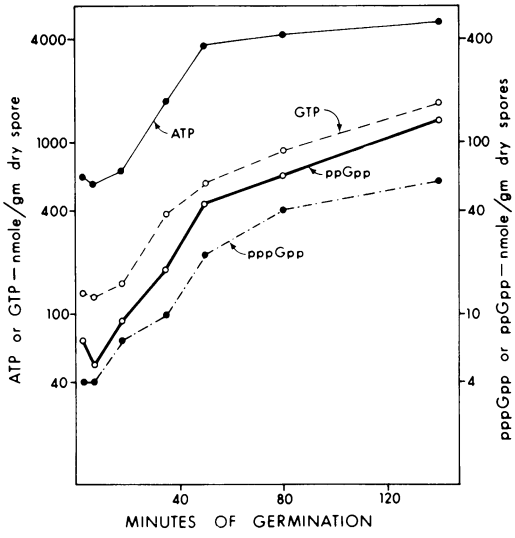

FIG. 1. *Levels of ATP, GTP, ppGpp, and pppGpp in germinating spores. Spores were germinated and extracted, and nucleotides were separated and quantitated as described.*

third minute of germination, as were high levels of ATP and GTP (Fig. 1) (22). After this initial rise, values for all four nucleotides were relatively constant in the first 15 to 20 min of germination, but at this time all began to increase (note different scales). The increase in ATP and GTP levels has previously been ascribed to the initiation of purine biosynthesis which begins at this time (22). It is significant that the ratio of ppGpp plus pppGpp to GTP remained relatively constant (between 0.07 and 0.11) from 3 to 120 min of germination in view of the 20- to 40-fold increase in this ratio accompanying the stringent response in *B. subtilis* (7). Indeed, the low ratio of ppGpp plus pppGpp to GTP in germinating spores was only slightly higher than that in unstarved vegetative cells of *B. subtilis* (about 0.05 [7]), although the relative amounts of ppGpp and pppGpp were different. These data are certainly consistent with the absence of any highly uncharged tRNA species during germination and strongly suggest that ppGpp and pppGpp are not involved in causing or regulating the rapid proteolysis which accompanies spore germination.

DISCUSSION

Constant specific acceptor activities of tRNA in dormancy and germination. Since total spore RNA content increases only 15% by 75 min of germination (19), it is not surprising that there were no large differences (>15%) in specific acceptor activities of tRNA from dormant and germinated spores. However, these data support the earlier suggestion (23) that the

5% of dormant spore RNA which is degraded in germination is not primarly tRNA, since this would have resulted in significant decreases (on the average 35%) in all specific acceptor activities upon germination (22, 23). The results also suggest that, if there are significant differences in levels of a few tRNAs between dormant spores and vegetative cells (up to 1.85-fold differences seen in *B. megaterium* KM [26]), these differences are not rectified during the first 75 min of germination either by synthesis or degradation of such specific tRNAs.

Accuracy of method for estimation of charged tRNA. Further conclusions derived from much of the data presented in this work will depend in large measure on the accuracy of the method used to estimate the amount of charged tRNA in vivo. That the method was indeed accurate is suggested by the high efficiencies of both recovery and deacylation of added aminoacyl-tRNAs (Table 4), as well as the effectiveness of periodate oxidation of deacylated tRNA (column 4, Table 5). It might, however, be argued that some values for high levels of charging of germinated spore tRNAs might not reflect conditions in vivo, but rather are artifacts generated by charging of tRNAs which took place after addition of trichloroacetic acid. There are two observations which argue against this possibility. First, several workers have utilized an identical or similar procedure for isolation and estimation of aminoacyl-tRNA in bacteria, and their results were consistent with the physiology of the cells in question (6, 12). These results included observation of very low levels of charging of some tRNAs. Second, and possibly more important, is the observation that from 3 to 120 min of germination spores have low levels of pppGpp and ppGpp (Fig. 1), levels similar to those in vegetative bacteria. It is known that the presence in vegetative bacteria of one or more species of uncharged tRNA will result in accumulation of high levels of pppGpp and ppGpp (2, 9, 15). Since this is not observed during germination, it strongly suggests that the high levels of charging of spore tRNAs throughout germination are not artifacts, and also suggests that tRNAs for the six amino acids I did not assay are also probably charged to a high level.

Significance of charged tRNA in dormant spores. The low level of charging of most tRNA species in dormant spores was not an unexpected finding in view of the small amount of ATP present. However, it was surprising to find high levels of charging of several tRNA species. Although I have no good explanation for this finding, it is possible that these aminoacyl-tRNAs serve some important control function in the breaking of dormancy, possibly by donating their amino acid to preformed proteins or other such acceptors. Indeed, such reactions have been observed in bacteria (13). However, it is equally possible that the few charged tRNAs present in dormant spores reflect either the aminoacylated species remaining when protein synthesis was halted during sporulation, or the greater stability of these particular species, or both.

Significance of invariant levels of tRNA charging in germinated spores. Although it was not surprising that charged tRNAs appeared by the first minutes of germination, it is significant that all species tested were highly charged and that there were no changes in the level of charging from 6 to 75 min of germination. These data suggest that neither low levels of tRNA charging nor changes in the level of tRNA charging are involved in regulating events taking place through 75 min of spore germination. Since both preexisting dormant spore protein and newly synthesized protein are rapidly degraded during this time (16, 17), it therefore follows that this intracellular proteolysis is not caused by a low level of charging of one or several tRNA species as has been observed in *E. coli* (8). This is made even more likely by the observation that this rapid proteolysis is ended by 75 min of germination (17) (P. Setlow, unpublished results, 1973). I cannot, of course, rule out regulation by either low levels of charging of tRNA species I did not assay, low levels of charging of a minor isoaccepting tRNA, or a difference in charging too small to have been detected. However, the low levels of ppGpp and pppGpp through 120 min of germination, as mentioned above, and the fact that protein synthesis occurs (11) are certainly consistent with the absence of any highly uncharged tRNA species in germinating spores.

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